#26

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent application of: Leadlay *et al.*,

Serial No: 09/214,453   Examiner: Kathleen M. Kerr

Filed: 5 January 1999   Art Unit: 1652

For: Polyketides and their synthesis Docket No:

DECLARATION UNDER 37 CFR §132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SIR:

I, Thomas James Simpson, hereby declare:

1) I am Professor Thomas James Simpson of 11 Caledonia Place, Clifton, Bristol BS8 4DJ,

and The School of Chemistry, University of Bristol, BS8 1TS, UK. I was born on the 23rd February,

1947 and I am a citizen of the United Kingdom. I studied at the University of Edinburgh from 1965 to

1969, gaining my BSc degree in Chemistry with First Class honours in 1969 and being awarded the

Macfarlan Smith prize for the candidate with the top marks in the Final Honours examination. I

studied at the University of Bristol from 1969 under the supervision of Professor Jake MacMillan

FRS, and was awarded the degree of PhD in 1973 on the basis of a thesis entitled 'Structural,

Stereochemical and Biosynthetic studies of Three Groups of Fungal Metabolites'. I was subsequently

awarded the degree of DSc in 1986 by the University of Edinburgh for a thesis entitled 'Biosynthesis

of Fungal Metabolites'. In the UK system the DSc degree is awarded on the basis of a substantial

body of independent published work. After postdoctoral research fellowships in the University of

Liverpool and the Australian National University (with Professor Arthur J Birch), I was award d a

1

SRC Research fellowship and subsequently an SERC Advanced Fellowship to carry out independent research in the University of Liverpool. I was appointed to a Lectureship in Chemistry at the University of Edinburgh in 1978, to the Chair of Organic Chemistry at the University of Leicester in 1988 and to the Chair of Organic Chemistry at the University of Bristol in 1990 where I am Head of Organic and Biological Chemistry. I am the recipient of the Royal Society of Chemistry Corday-Morgan Medal and Prize (1984), Tilden Lectureship (2001) and Simonsen Lectureship (2003). I was Vice President of Perkin Division, the organic chemistry division, of the Royal Society of Chemistry (1993-1995). I was elected a Fellow of the Royal Society in 2001.

2) I have carried out research on the chemistry of natural products for 33 years. This work has covered all aspects of the structure elucidation, synthesis and biosynthesis of plant and microbial metabolites. My research has largely dealt with natural products of the polyketide pathway and over the last twelve years this work has concentrated on the enzymology and molecular genetics of compounds of fungal and bacterial origins. I have published extensively on the structural and biochemical characterisation of proteins from Type II polyketide synthases and I also have active research programmes on other areas of polyketide research, namely fungal Type I PKSs, bacterial Type I PKSs, and chalcone and stilbene synthases from higher plants. I have a total of 154 publications in these and related areas, and I have lectured extensively in the UK, Europe, North America and elsewhere on these topics. I was a Member and subsequently Chairman of the Editorial Board of 'Natural Product Reports' for 18 years from 1984 until 2002. I have chaired the organising committees of three major international conferences on the chemistry, biochemistry and molecular genetics of polyketide, fatty acid and non-ribosomal peptide synthases in the last seven years.

3) I have read US5962290 ("Khosla '290") and the specification of the above-identified application, initially published as WO98/01546.

2

4) I am familiar with the state of the art at the priority date of the above-identified application, nam ly 5 July 1996. Specifically I have reviewed the documents in the list of r ferences annexed hereto.

5)   I am commenting on the teaching of Khosla '290 and the differences between this teaching and the invention disclosed in the above identified patent application.

6) The polyketide pathway is widespread in nature, being found in fungi, bacteria, marine organisms and higher plants where it is responsible for the production of a vast array of complex structures from simple aromatic compounds like 6-methylsalicylic acid to complex non-aromatic compounds such as rapamycin. Many of these compounds display important biological activities, such as antibiotics (e.g. tetracyclines, erythromycin), anti-cancer agents (daunomycin and dynemycin A), antiparasitics (avermectins and monensin), antifungals (strobilurins and amphotericin), immunosuppressive agents (FK506 and rapamycin), and cholesterol lowering agents (lovastatin) (O'Hagan, *The polyketide metabolites*, Ellis Horwood, Chichester, 1991). Despite their great structural variety, all of the polyketides are related by their common biosynthetic origins. They are derived from highly functionalised carbon chains whose assemblies are controlled by multifunctional enzyme complexes: polyketide synthases (PKSs). PKSs catalyse a repetitious sequence of decarboxylative condensation reactions between simple acyl thioesters. Each condensation reaction may be followed by a sequence of modifying reactions: ketoreduction, dehydration and enoyl reduction which modifies the oxidation level of the chain. The most common acyl esters utilised by PKSs are acetate and propionate, which are used in their carboxylated forms, malonate and methylmalonate, when acting as chain extending units. Certain PKSs can however introduce additional structural complexities by using a wide variety of chain starters besides acetate and propionate: *e.g.* C4 and C5 branched and cyclic carboxylic acids, benzoate, cinnamate and various amino acids; and a range of chain extender units, malonate, methylmalonate, ethylmalonate; and by introducing branching methyls from *S*-adenosyl methionine. PKSs can also control the overall chain lengths and the number and mode of

3

cyclisation reactions. Importantly the basic carbon skeletons produced and released by the PKS can then be further modified to lesser or often greater xtents by downstream enzymes. The range of these secondary modifications may encompass a very wide range of standard enzyme mediated process s familiar to those trained in the art of natural products chemistry. It is these different possibilities in both chain synthesis and subsequent elaboration that are responsible for the huge range of polyketide structures found in nature.

7) The genetics and enzymology of polyketide biosynthesis has been an area of active research since the mid 1980s. Much of this arose from the isolation of the biosynthetic genes for the polyketide actinorhodin from *Streptomyces coelicolor* in 1984 (Malpartida and Hopwood, *Nature*, 1984, **309**, 462-464). Importantly these genes were shown to be clustered and were shown to consist of a series of separate ORFs that encoded single essentially mono-functional enzymes. In bacteria two distinct types of PKS were known at the effective date (5th July 1996). Type II PKSs are responsible for biosynthesis of the aromatic polyketides, such as actinorhodin, frenolicin, tetracycline, and daunorubicin. Type I PKSs are responsible for biosynthesis of complex highly reduced polyketides, such as erythromycin, rapamycin, tylosin, avermectin and amphotericin. These two types of PKS were known to have very different structures (*vide infra*) and to act in different ways to produce intermediates with markedly different structures and properties. Type II PKSs produce highly oxygenated poly-beta-ketoacyl chains, mainly acetate-derived, which experience little or no reductive modification during their assembly. It was well known to those skilled in the art that such intermediates are highly labile chemically (Harris and Harris, *Pure & Appl. Chem.*, 1986, **58**, 283-294) and that an essential function of the PKS must be to stabilise these intermediates and prevent uncontrolled cyclisations and other spontaneous chemistry occurring (Harris and Wittek, *J. Am. Chem. Soc.*, 1975, **97**, 3270). In contrast, the highly reduced intermediates characteristic of Type I modular PKSs display a much greater intrinsic stability and prevention of spontaneous chemistry is not a significant issue. The range of chemical modifications normally seen in Type II PKS-mediated chain ass mbly is rather limited and much of the important structural variety observed for these

4

compounds results from post-PKS modifications. In contrast, the range of structural variation that may be controlled by Type I PKSs is very large and the structures of Type I PKS products are normally much closer to th core structure produc d by the PKS. Whil the variety of downstream modifications is potentially wide, the observed degree/extent of post-PKS modification is normally much less than that observed with Type II PKS derived compounds. Thus the potential value of Type I PKSs for generating novel structural variety was intrinsically much higher than for Type II PKSs. This greater potential was recognised by those trained in the art even before detailed knowledge of the structures of the PKSs became available.

8) The first Type II PKS gene cluster, for actinorhodin from *Streptomyces coelicolor*, was isolated and heterologously expressed in 1984 (Malpartida & Hopwood, *Nature*, 1984, **309**, 462-464). The first gene sequence information was reported in 1989 and by the effective date gene sequences for numerous Type II PKSs from different *Streptomyces* species had been identified and characterised, establishing the basic composition of Type II PKSs (Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238). In Type II PKSs the essential enzyme activities are found as single, mono-functional proteins (Hopwood & Sherman, *Annu. Rev. Genet.* 1990. **24**, 37-66; Katz & Donadio, *Annu. Rev. Microbiol.* 1993, **47**, 875-912; Shen & Hutchinson, *Science* 1993, **262**, 1535-1540). They are responsible for the synthesis of a poly-beta-ketoacyl thioester of a given length, typically but not exclusively 16-24 carbons; controlled folding, cyclisation and aromatisation to form one or more aromatic rings; and release of the end product from the PKS. The chain may be subject to reduction of one (rarely more) of the ketonic moieties and methylation on carbon (very rarely) or oxygen. Two proteins had been unequivocally shown to be essential in the chain assembly process. These were a beta-keto-acyl synthase (KS) and an acyl carrier protein (ACP). A third component was surprisingly found also to be necessary. This protein has high overall sequence similarity to the KS, but lacks the essential active site cysteine found in all known condensing enzymes. At the priority date no analogous protein was known in other Types of PKS. It was called the Chain Length Factor (CLF) on the basis of a series of *in vivo* experiments, *vide infra*, the results of which were interpreted as

5

showing that the CLF determined the overall length of the chain produced by the Type II PKS (McDaniel *et al, Science*, 1993, **262**, 1546-1550). This set of three components was shown *in vivo* to be the minimum required to produce a stable isolable polyketide compound and as such they have been dubbed the 'minimal' PKS (McDaniel *et al, Science*, 1993, **262**, 1546-1550; McDaniel *et al., Proc. Natl. Acad. Sci. U.S.A.* 1994, **91**, 11542-11546). The components of the minimal PKS are used for every round of polyketide chain assembly and so must be able to handle chains of increasing length, although their chemical nature is relatively unchanged. The minimal PKS of every natural Type II PKS system catalyses an essentially identical series of reactions, since the starter is almost always acetate and the extender units malonate. Closely associated with the minimal PKS genes a number of specific additional activities may be found as part of the biosynthetic gene cluster. As anticipated by those skilled in the art (Harris and Wittek, *J. Am. Chem. Soc.*, 1975, **97**, 3270) these include cyclases and aromatases whose role appears to be control of cyclisation and aromatisation of the highly labile polyketide chain, so-called "ketoreductases" which bring about reductions of ketone-derived functionality and occasionally methyl transferases which introduce a methyl branch onto the polyketide or convert phenolic hydroxyls into methyl ethers. The timing of the reductions, cyclisations and aromatisations relative to chain assembly remain uncertain. This is exemplified by the "ketoreductase". Khosla '290 describes the role of these as being to bring about highly regiospecific reductions of the fully assembled poly-beta-ketide chain. However, earlier sequence comparisons with known reductases revealed a higher homology to those which are known to effect reduction of polyhydroxy aromatic rings (Vidalcros *et al., Eur. J. Biochem.*, 1994, **219**, 985-992), than to typical type I PKS or fatty acid synthase KRs. It was also known in the art that such poly-hydroxy-aromatic rings are subject to facile chemical reduction with mild reducing agents such as sodium borohydride (Bycroft *et al., J. Chem. Soc., Chem. Commun.*, 1974, 443). This information suggested to those skilled in the art that these Type II PKS KRs were likely to effect reduction of intermediates formed *after* cyclisation and aromatisation of the fully or partially assembled poly-beta-ketide intermediate, reinforcing the perceived differences between chain extension on Type II and Type I PKS (Cane, *Science*, 1994, **263**, 338-340). The difficulties encountered in assigning biochemical function to the

6

component enzymes at that time are similarly illustrated by the fact that the 'CLF' has since been shown to act as a decarboxylase responsible for decarboxylating malonyl-ACP to produce the (acetyl-ACP) primer for initiating polyketide chain assembly (Bisang *et al.*, *Nature*, 1999, **401**, 502-505), and is thus now more commonly referred to as KSβ, with the KS (condensing enzyme) becoming known as KSα. Although there was known to be no covalent linkage of the individual Type II PKS components, they were thought to associate in some way to form the functional complex. The KS and CLF genes were known to be transcriptionally linked and to be expressed in equimolar amounts and it was believed that their products associated to form a tightly bound heterodimer. The overall stoichiometry of the minimal PKS and the nature of the interactions in the complex were and remain uncertain but the aromatase/cyclase components have also been shown to exert direct influence on polyketide chain length (Petkovic *et al.*, *J. Biol. Chem.* 1999, **274**, 32829-32834), further strengthening their assignment to playing a role in the structural integrity of the PKS complex. In contrast to all other related synthases, the Type II PKS gene clusters uniquely lack any identifiable malonyl transferase genes and so the identity of the essential transferase activity that loads malonyl units from malonyl CoA onto the ACP was not known at the effective date and remains a matter of some continuing debate. Acyltransferases have been found infrequently in some Type II PKS clusters but these are invariably associated with the relatively rare systems which utilise starter units other than acetate (Moore and Hartwick, *Nat. Prod. Rep.*, 2002, **19**, 70- 99).

9) Khosla '290 mainly concerns itself with two issues. The first is the construction of a strain of *Streptomyces coelicolor*, designated CH999, from which the *act* PKS gene cluster has been almost entirely deleted. This was then used as host for a shuttle vector, pRM5 and derivatives thereof, which was used for transfer of homologous and heterologous Type II PKS genes into the CH999 host strain. In terms of prior art, pRM5 is derived from the known SCP2 low copy number plasmid (Lydiate *et al.*, *Gene*, 1985, **35**, 223-235) which was known to be effective for *in vivo* transformations of *Streptomyces* spp. Plasmid pRM5 contains actinorhodin PKS components under the control of the

7

native (*S. coelicolor* deriv d) promoter for the *actI/actIII* genes, and the cognate activator protein ActII-ORF4 (see McDaniel *et al.*, *Science* 1993, 262, 1546-1550).

Second, Khosla '290 proceeds to describe the replacement of the various *act* genes in pRM5 with Type II PKS genes from other sources followed by transformation of the host strain CH999 with these new plasmid vectors resulting in production of hybrid Type II PKSs. These experiments are exemplified by examples 3-6, which largely reiterate results revealed in previously published work (reviewed in Katz & Donadio, *Annu. Rev. Microbiol.* 1993, 47, 875-912, see page 891 and references therein; Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, 49, 201-238; McDaniel *et al.*, *Science*, 1993, 262, 1546-1550; McDaniel *et al.*, *J. Am. Chem. Soc.* 1993, 115, 11671-11675; McDaniel *et al.*, *Proc. Natl. Acad. Sci.* U.S.A. 1994 91 11542-11546; McDaniel *et al.*, *J. Am. Chem. Soc.* 1995, 117, 6805-6809; McDaniel *et al.*, *Nature*, 1995, 375, 549-554; Fu *et al.*, *Biochemistry* 1994, 33, 9321-9326, Khosla *et al.*, *Mol. Microbiol*, 1992, 6, 3237-3249, Khosla *et al.*, *J. Bacteriol.* 1993, 175, 2197-2204) As anticipated by the prior art (Bartel *et al. J. Bacteriol.*, 1990, 172, 4816-4826, Strohl *et al.*, 1989, *In Genetics and Molecular Biology of Industrial Microorganisms* Ed., Hershberger, Queener & Hegeman p68-84), many but not all of these hybrid PKSs were functionally active and effected the production of a series of new aromatic polyketides. Structural analysis of the resultant structures were used to propose a set of 'design rules' for production of predicted structures by hybrid Type II PKSs (McDaniel *et al.*, *Nature*, 1995, 375, 549-554). The structures produced by these hybrid PKSs resulted from two processes. The first of these is the assembly of the poly-beta-ketide chain of a specific length and its initial cyclisation by the minimal PKS. Depending on the other components present in the hybrid PKS, further enzyme mediated reductions, cyclisations and aromatisations may occur. The remainder of the observed structure reflects spontaneous chemistry which occurs on the labile parts of the structure revealed on release from the PKS. While there is a degree of predictability in the type of substructures which will form as a result of such spontaneous chemistry (Harris and Harris, *Pure & Appl. Chem.*, 1986, 58, 283-294) which was well known to those familiar with prior art in the area, formation of these substructures are not subject to facil external control. Claim 4 (a) (iii), in

8

particular, makes reference to a method for the use of heterologous combinations of KS and CLF, specifically to determin the length of chain assembled or in effect the number of condensation cycles controlled by the hybrid PKS. However it was evident to those skilled in the art and especially from Khosla et al.'s own earlier publications recited above that routinely such combinations failed to yield an active hybrid PKS and so no production of aromatic polyketides results. It is also noteworthy that there is no disclosure whatsoever in Khosla '290 to provide heterologous loading modules lacking a KS domain to facilitate production of novel and structurally distinct analogs of known polyketides.

10) In marked and surprising contrast to the situation with Type II PKSs, genetic analysis first reported in 1990 showed that the biosynthesis of 6-deoxyerythronolide B, the aglycone of erythromycin, is controlled by three very large multifunctional proteins, DEBS 1-3, each of which contained two condensation modules (Cortes *et al.*, *Nature*, 1990, **348**, 176-178; Donadio *et al.*, *Science*, 1991, **252**, 675-679; Bevitt *et al.*, *Eur. J. Biochem.*, 1992, **204**, 39-49). As shown in that reference, each condensation module in turn contains domains corresponding exactly to the required functionalities: ketosynthase (KS), acyl transferase (AT), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) needed to produce the oxidation level observed for the part of the polyketide chain corresponding to that module, the core minimum requirements being KS, AT and ACP. The second module of DEBS3 (module 6) is terminated by a thioesterase domain (TE) whose function is to release the final heptaketide product from the PKS and, presumably, to bring about macrolactone formation. Thus the synthase, now termed a modular Type I PKS, has a specific catalytic function for every individual chemical step required to produce the macrolide product. The modules within the PKS are arranged in the exact sequence required to achieve the observed oxidation level. Thus the successful production of 6-deoxyerythronolide B required the correct functioning of a very large number of different active sites, each housed in an individual enzyme domain. This is in complete contrast to Type II PKSs which contain only one enzyme for each type of reaction catalysed by the synthase, each enzyme being used repeatedly as necessary. Module 1 begins with two additional AT and ACP domains which were assumed to be responsible for

9

priming the PKS with starter propionate unit (Donadio *et al.*, *Science*, 1991, **252**, 675-679; Aparicio *et al.*, *J. Biol. Chem.*, *1994*, **269**, 8524-8528). This is now recognised in the above-identified application (WO98/01546) to represent an autonomous 'loading module' which selects the appropriate starter acyl unit and transfers it to the first condensation module. This was not recognised or commented on in Khosla '290. The next major advance in sequencing Type I PKSs came in 1995 with the publication of the rapamycin PKS gene sequence (Schwecke *et al.*, *Proc. Natl. Acad. Sci.*, *USA*, 1995, **92**, 7839). This showed a number of surprising and interesting differences with the DEBS structure. Again the synthase comprises three extremely large multifunctional proteins and there is an exact correspondence between the number of modules and the number of required condensations (14 in this case) but the number of condensation modules in each protein varied from 4 in RAPS3, 5 including a distinctive loading module in RAPS1, to 6 in RAPS2. The TE is replaced by a separate protein dubbed PIE analogous to the peptide forming modules of non-ribosomal peptide synthases and assumed to be responsible for the observed fusion of the polyketide product via an amide linkage to pipecolic acid. Two other significant comparisons were: (i) the observation of a number of apparently functional but redundant KR, DH and ER domains which are not utilised in generation of the observed functionality in the polyketide; and (ii) the presence of AT domains which were selective for either malonate or methylmalonate as the chain extending acyl unit. These have diagnostic active site motifs that allow them to be distinguished.

11) Based on extensive studies of the DEBS PKS there was strong evidence for the overall structure adopted by the modular Type I PKSs. Each of DEBS 1-3 associated to form a tight dimer (Caffrey *et al*, *FEBS Lett*. 1992, **304**, 225-228; Aparicio *et al.*, *J. Biol. Chem.*, 1994, **269**, 8524-8528) with each dimer having the core KS, AT, ACP arranged to form a head to head double helix with the accessory KR, DH and ER domains on the periphery of the helix. There appeared to be a weaker but specific interaction between the terminal ACP of one protein *e.g.* ACP2 of DEBS1 with the initial KS of the subsequent protein *e.g.* KS3 in DEBS2 (Staunton *et al.*, *Nat. Struct. Biol.* 1996, **3**, 188-192).

10

The domains in each of the large DEBS proteins are separated by distinctive linker regions which have been shown to be amenable to selective proteolysis.

12) Prior to the effective date, directed genetic manipulation experiments guided by sequence information to produce modified erythromycins with predictable structural modifications, were strictly limited. Mutations of the KR domain in module 5 of DEBS3 produced a macrolide in which a ketone function at 5 was retained and mutation of the ER in module 4 gave an unsaturated analogue in which the normal reduction of the C6-C7 double bond had not occurred (Donadio *et al., Science*, 1991, **252**, 675; Donadio *et al., Proc. Natl. Acad. Sci, USA*, 1993, **90**, 7119-7123). However an analogous experiment on the DH in module 4 gave no identifiable products despite only one residue undergoing a conservative alteration (Bevitt *et al., Biochem. Soc. Transactions*, 1992, **21**, 30S). Khosla '290 describes published work (Kao *et al., Science*, 1994, **265**, 509-512) in which DEBS 1- 3 was expressed in *S. coelicolor* CH999 to produce the natural erythronolide product and also the nor-analogue in which the natural propionate starter has been replaced by acetate, presumable reflecting the greater availability of acetate as a substrate in this strain, an interpretation which was supported by the observation that yields of the natural macrolide were increased by supplementation of the growth medium with propionic acid. While the ability to produce structural variety is of interest it should be noted that to practitioners in the field such mixtures are undesirable due to the problems associated with separation and purification of closely related structures. Similar expression of DEBS1 on its own resulted in production of a triketide lactone (TKL) with a propionate primer in very low yields. However, this was anticipated in prior work by Leadlay and co-workers (Leadlay *et al., Society for General Microbiology meeting*, Warwick, January 1994, quoted by Hutchinson (at p.378) in Hutchinson, *Biotechnology*, 1994, **12**, 375-380; Wiesmann *et al., Abstracts of International Symposium on the Biology of Actinomycetes*, July 1994, Moscow, 154), in which expression in the normal host *Saccharomyces erythraeus* of DEBS1, which had been specifically engineered to place the TE from DEBS3 after ACP2 to give the DEBS1-TE construct, gave the TKL in high yields. This demonstrates the importance of the presence of the TE which results in much improved yields

11

presumably du to the catalysed removal of the polyketide intermediate rather than relying on th spontaneous release in the absence of the TE domain. Khosla '290 reiterates subsequent work (Kao *et al.*, *J. Am. Chem. Soc.*, 1994, **116**, 11612-11613), on expression of a similar DEBS1/TE construct in the CH999 host. In agreement with the previous result, the presence of the TE gave much improved yields of TKLs but again a mixture of acetate and propionate primed products was produced. In an extension of this work, Khosla '290 reports an experiment in which the TE was repositioned at the end of ACP5 with the production of the anticipated hexaketide lactone from the resulting truncated polyketide intermediate.

13) To summarise what this prior art would have taught a person skilled in the art with regard to the feasibility of producing novel compounds by rational engineering of type I PKSs at the effective date the following points should be noted:

- Knowledge of the structures of Type II and modular Type I PKSs indicated that they were very different in structure at the protein level, the former consisting of a labile complex in which a limited number of essentially monofunctional proteins are associated with one another, whereas the latter consists of a tight, defined dimeric structure of extremely large, highly multifunctional, multidomain proteins.

- The Type II PKS components are used iteratively in each chain elongating cycle whereas modular Type I PKSs have a separate catalytic domain for each and every step in the chain elongation process.

- Type II PKSs produce poly-beta-ketide intermediates which are highly labile and so must be stabilised by interactions, which remain non-defined, with the PKS proteins. They show little or no reductive modification, and it is likely that reductive modification occurs post aromatisation at a defined site or sites. The scope for structural variation arises from the putative ability to alter chain length and the number and mode of cyclisation and aromatisation reactions. The control of these processes was poorly understood at the effective date and remains so.

12

- Modular Type I PKSs, in contrast, produce highly functionalised chains which display great potential for structural variation through control of starter group selection, but also through variation in chain l ngth, variation in selection of extender unit to remove or introduce methyl (and ethyl) branches, variation in oxidation level from methylene through to alkene, secondary alcohol to ketonic functionality, control of hydroxyl and methyl/alkyl branch stereochemistry. These are all exemplified in the embodiments in the above-identified application (WO98/01546).

- The environment of the host and in particular acyl substrate availability has an important influence on the structures produced by the expressed PKS.

14) At or before the priority date, the person skilled in the art would have been well aware of the desirability of obtaining active hybrid Type I PKSs so that novel complex polyketides would be produced, since such products were perceived as intrinsically much more useful and desirable than the aromatic compounds disclosed by the prior art and by Khosla '290. The prior art including Khosla '290 is silent regarding the independent identity and functionality of the loading module (which lacks a KS domain) and the desirability of producing constructs with heterologous loading modules to facilitate production of a range of novel polyketides, such as erythromycin analogues or triketide lactones with improved or altered biological or physico-chemical properties as disclosed in the present application.

All alterations in Type II PKS systems were made by a "mix and match" strategy involving entire genes. It was not obvious how this could be adapted to the giant multifunctional gene products of the Type I PKS systems. The manipulation of homologous Type I PKS systems was limited to a single example of a Type I PKS and to the methods of deletion or point mutation, neither of which could be applied to making the desired functional hybrids. It did not occur to anyone prior to the disclosure made in the present application that the precise *in vitro* splicing together of portions of Type I PKS DNA derived from one or more natural PKS genes together with portions of an acceptor Type I PKS would give rise to a functional hybrid. Furthermore, it was not clear until the experim nts were

13

attempted whether deleterious changes in protein folding and structure introduced by such genetic alterations would lead to failure of the dimeric PKS proteins to associate to form a functional hybrid, or whether structural alterations introduced into the polyketide intermediates would lead to their not being recognized and processed correctly by subsequent domains as substrates for chemical modification, transfer and final release from the synthase.

15) All prior art taught the informed practitioner that Type II PKSs and modular Type I PKSs were remarkably different and so there was no reason to infer that prior art teaching on Type II PKSs would enable the skilled person to modify Type I PKSs to generate functional hybrids. This is a distinction that is not addressed in "Khosla '290, which deals in the main with Type II PKSs. Prior art and Khosla '290 give no adequate precedent for the construction of hybrid modular PKSs. Indeed, the well-known lack of success in constructing hybrid minimal type II PKSs, i.e. ones in which the KS and CLF subunits were derived from different PKS gene clusters, meant that the problems associated with achieving productive interactions among heterologous proteins were well-recognized. Khosla '290 teaches only that modular Type PKSs can be heterologously expressed in their entirety or in truncated form. In all cases cited in Khosla and in the prior art there is no teaching on the methods of construction or expression of hybrid modular Type I PKSs. Indeed prior art points to the low level of success in alterations involving even homologous Type I PKS genes. The matter in Khosla '290 points

14

to the importance f having a suitable host environment with suitable substrate availability for efficient polyketide production, but they do not recognise this as a problem or give any guidance on how this problem should be solved.
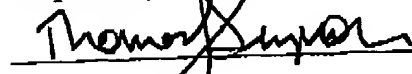
16) It is my opinion that neither prior art nor the disclosures in Khosla '290 provide evidence for lack of novelty for hybrid Type I polyketide genes having loading modules which lack ketosynthase activity. Furthermore, Khosla '290 clearly fails to appreciate the advantages utilizing such loading modules as set forth at pages 128 and 129 of the present application.

17) In my opinion therefore, amended claim 67 and claims dependent therefrom represent an advance in the art of generating Type I polyketides of improved or altered biological or physico-chemical properties. Accordingly, constructs presently claimed are considered to merit patent protection.

18) I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on the information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such wilful statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: 13th June 2003

Professor Thomas J. Simpson

15